

## Immunization against experimental rabbit cysticercosis using liposome-associated antigen preparations

P. S. CRAIG<sup>1</sup> and O. ZUMBUEHL<sup>2</sup>

<sup>1</sup>Department of Parasitology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, England

<sup>2</sup>Physikalische Pharmazie-ETH, Sonneggstrasse 5, CH-8092 Zurich, Switzerland

### ABSTRACT

Rabbits were vaccinated once, by subcutaneous and intradermal injection, with sonicates of oncospheres (TpO) or conditioned media from *in vitro* maintained mature metacestodes (TpMcES) of *Taenia pisiformis*. Extracts were either incorporated into or mixed with unilamellar liposomes (reverse phase evaporative vesicles) or emulsified in Freund's Incomplete Adjuvant (FIA). Control groups received liposomes or FIA without antigen, or antigen preparation without adjuvant. Rabbits were challenged orally two weeks after vaccination with approximately 1500 eggs of *T. pisiformis* and necropsied eight weeks after challenge. A mean of 155 cysts was recovered from seven control rabbits. A 67% reduction in peritoneal cyst numbers was obtained in TpO–IFA vaccinated rabbits compared to 75% for the TpO–liposome entrapped group. The highest level of protection (86%) was obtained when TpO was mixed with but not entrapped in liposomes. Only 32% and 39% reduction in peritoneal cyst numbers was obtained after immunizing with the TpMcES preparation in liposomes or IFA respectively, however >85% of peritoneal metacestodes were dead (necrotic or calcified) and suggests a different immune response than occurs after vaccination with oncosphere extracts. Specific anti-oncospherical or anti-metacestode ES antibody (IgG) responses at two weeks post vaccination were similar in rabbits immunized with liposome or IFA associated extracts.

KEY WORDS: *Taenia pisiformis*, immunization, oncosphere, metacestode, liposomes, rabbits

### INTRODUCTION

The ability to immunize animals against taeniid larval cestode infection using living oncospheres or their extracts (sonicates or *in vitro* culture 'ES' preparations) has been convincingly demonstrated to varying degrees in mice, rabbits, sheep and cattle (see reviews, FLISSER *et al.*, 1979; RICKARD, 1982; RICKARD & WILLIAMS, 1982). However, in almost all cases where oncospherical antigen preparations were used as experimental vaccines, they were associated with an adjuvant, notably Freund's Complete (FCA) or Incomplete (FIA) adjuvants. Unfortunately Freund's adjuvant is only acceptable for use in experimental animals and is definitely not acceptable for human use due largely to problems of granuloma formation, plasma cell tumours (in mice) and mineral oil persistence (refs cited by SIDDIQUI *et al.*, 1981).

There is therefore a need to investigate alternative ways of presenting immunizing antigens to the host, especially if practical vaccination against human cysticercosis (*Taenia solium*) or human hydatidosis (*Echinococcus granulosus* and *E. multilocularis*) ever becomes a feasible proposition. The use of liposomes could provide a useful and acceptable alternative to mineral oil based adjuvants.

Liposomes, originally described by BANGHAM *et al.* (1965) are microvesicles artificially composed of phospholipid membranes enclosing an aqueous compartment, which have remarkable similarities to biological membranes and exhibit immune adjuvant properties (TOM, 1980). Liposomes are readily taken up by macrophages and thus the liver is an important target organ. Most applications to date of liposomes in parasitology have involved targetting chemotherapy of protozoal infections (CROFT, 1986).

Here we report the use of liposomes to immunize rabbits against oral infection with *T. pisiformis*, a natural taeniid of rabbits. The results were compared with the use of Freund's Incomplete Adjuvant.

## MATERIALS AND METHODS

### *Rabbits and vaccination protocol*

Thirty-two, 16 week old female NZ white rabbits were divided into vaccination and control groups (see Table I). Rabbits were vaccinated intradermally and subcutaneously 0.5 ml total volume (approximately 300 µg protein) on Day 0, challenged orally two weeks later with approximately 1500 eggs per rabbit of *T. pisiformis* and necropsied eight weeks after challenge. The numbers of cysts in the peritoneal cavity (including liver surface) were counted and their viability noted macroscopically. Control groups were injected with FIA or liposomes without antigen or with immunizing antigen alone. Rabbits were ear bled at various intervals and sera stored at -30°C until used.

### *Immunizing extracts of T. pisiformis*

Oncospheres were artificially hatched from eggs of *T. pisiformis* and purified on Percoll (Pharmacia) to remove shell components and unhatched eggs (RAJASEKAR-IAH *et al.*, 1980), and sonicated in 0.15 M phosphate buffered saline pH 7.2 (PBS) as described by CRAIG & RICKARD (1981). The oncospherical sonicate was centrifuged at 500 g for 30 mins at 4°C and the supernatant used as the immunogen (TpO). Each rabbit was vaccinated with the product of approximately 75 000 oncospheres.

An excretory-secretory (ES) antigen extract was prepared from the mature metacystode stage maintained *in vitro*, essentially as described elsewhere (CRAIG, 1984). Briefly, 'conditioned' serum-free media (DMEM + 20 mM hepes + 1% glucose) was harvested every third day from 20 metacystodes in a 10 ml culture, pooled and concentrated through a 10 000 Mwt filter (Amicon Corp) and designated TpMcES.

### *Liposomes*

Unilamellar reverse phase evaporative vesicles (REVs) were prepared essentially as described by SZOKA & PAPHADJOPOULOS (1978). 20 mg sphingo-

**TABLE I.** Mean numbers of cysts of *Taenia pisiformis* present in peritoneal cavity of vaccinated or control rabbits

Vaccination group	No. rabbits	No. cysts		% cysts necrotic mean	% protection
		Range	Mean ± S.D.		
Control (lip) (IFA)	7	64-310	155 ± 90	8	—
TpO alone	4	49-80	62 ± 14	30	60
TpO+lip	4	22-80	39 ± 18 <sup>a</sup>	50	75
TpO+IFA	4	34-73	52 ± 19	80	67
TpO+lip ("free")	4	12-43	22 ± 14 <sup>b</sup>	10	86
TpMcES alone	3	74, 95, 165	111 ± 48	85	28
TpMcES±lip	3	60, 105, 150	105 ± 45	95	32
TpMcES±IFA	3	53, 88, 140	94 ± 44	100	39

Students t-test: b vs a— $p < 0.05$ . lip=liposomes. IFA=Incomplete Freund's Adjuvant. TpO=*T. pisiformis* oncosphere extract. TpMcES=*T. pisiformis* metacystode "ES" extract.

myelin (Sigma) and 8 mg cholesterol (recrystallized) were dissolved in 3 ml chloroform in a stoppered glass test tube suspended in a 37°C water bath. To this was added 3 ml of ether and then 1 ml of antigen and the mixture bath sonicated (at 37°C). Solvent was removed by rotary evaporation at 37°C for 30 mins. After vortex mixing REVs (liposomes) were produced.

In addition, liposomes were prepared exactly as above but without antigen ('empty') and used as controls. Also a batch of 'empty' liposomes were incubated in antigen solution at room temperature for 1 hr (i.e. antigen not entrapped in liposomes).

### *Freund's Adjuvant*

The TpO and TpMcES antigen preparations were emulsified in equal volumes of Freund's Incomplete Adjuvant (Sigma)

### *Enzyme linked immunosorbent assay (ELISA)*

The method of ELISA for detecting specific IgG antibodies to the TpMcES and TpO antigen preparations was essentially as described previously (CRAIG & RICKARD, 1981; CRAIG, 1984). Antigens were used to coat plates at ~ 5 µg prot/ml and all incubations carried out at 4°C. Rabbit sera were used at a dilution of 1:150.

## RESULTS

The mean numbers of cystic metacestodes obtained from the peritoneal cavity of rabbits in vaccinated and control groups is shown in Table I. A mean of 155 cysts per rabbit (range 64–310) were counted in the control group (FIA or liposomes without antigen) and approximately 92% appeared viable. Vaccination of rabbits with oncospherical sonicate TpO entrapped inside liposomes resulted in a 75% reduction in cyst numbers. This was comparable to the 67% reduction in cyst numbers obtained when TpO–FIA was used to immunize rabbits. However the greatest degree of protection, 86% was obtained in the group vaccinated with liposomes which had only been passively incubated in the TpO preparation, i.e. the antigen was not entrapped within the liposomes. Vaccination with TpO alone resulted in 60% protection.

In contrast, the level of protection obtained when rabbits were vaccinated with the TpMcES preparation, either in liposomes or FIA or alone was much lower, i.e. 32%, 39% and 28% respectively. However, a greater proportion of the cysts (85–100%) observed in the TpMcES vaccinated rabbits were dead, necrotic and/or partially calcifying, compared to the TpO vaccinated groups (Table I).

Measurement of specific IgG antibody responses using TpO or TpMcES antigen preparations in the ELISA revealed very similar responses in rabbits for both liposome or FIA associated vaccine groups (Fig. 1). There was perhaps some indication that anti-TpO antibody responses were maintained for a longer duration in the TpO–FIA group compared to the TpO-liposome group.

## DISCUSSION

A significant degree of protection (75% and 86%) against oral challenge with *T. pisiformis* eggs was obtained in rabbits immunized with a crude low speed sonicate of *T. pisiformis* oncospheres entrapped in, or passively coated onto liposomes (REVs). This level of protection was at least as good as that obtained when the oncospherical sonicate (TpO) was injected after being emulsified in Freund's Incomplete Adjuvant (i.e. 67%). RAJASEKARIAH *et al.* (1985) used similar TpO

FIG. 1.

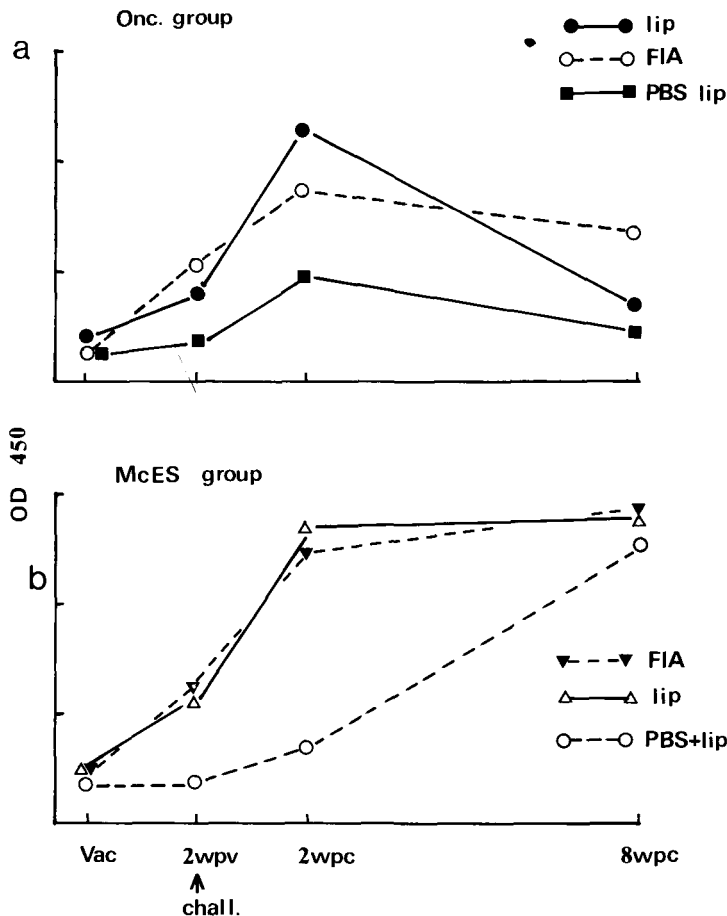


FIG. 1. Serum antibody responses (IgG-ELISA) against a) TpO or b) TpMcES extracts in groups of rabbits vaccinated by a) oncospherical or b) metacystode (McES) extracts in liposomes or FIA, or PBS 'controls' in liposomes. wpv=weeks post vaccination; wpc=weeks post challenge.

sonicates to vaccinate rabbits, and obtained 90% protection, after two intramuscular injections two weeks apart in Freund's Complete Adjuvant followed by FIA respectively. In the present study the greatest level of protection (86%) was achieved after a "single" injection with TpO coated onto the surface of liposomes. This level of protection was greater than the 60% level obtained after vaccination of rabbits with TpO alone or the 76% level obtained when the TpO preparation was entrapped inside liposomes. This suggests that functional antigen in TpO may be better presented to the immune effector cells, e.g. macrophage on the liposome surface. Interestingly VAN ROOIJEN & VAN NIEUWMEGEN (1980) observed a similar result when they measured the immune response in rabbits against human serum albumin (HSA) that had been coated on 'empty' liposomes or entrapped in the vesicles. The greatest responses were associated with liposome surface coated HSA, and these authors suggested that adjuvant effects of liposomes are mediated by antigen exposed on the liposome surface.

In contrast to the immunity obtained by TpO vaccination against invading oncospheres from the oral egg challenge, which is probably effective in rabbits 3-5 days after infection (RAJASEKARIAH *et al.*, 1985), it appears from the present study

that a different immune response is active in the TpMcES vaccinated rabbits and is directed at the later metacystode stages. This was manifested by the presence of large numbers of dead or necrotic larvae in the peritoneal cavity. These observations fit in with the concept of post-encystment immunity, where the immune response affects the mature or established parasite rather than the early oncosphere stage (see reviews by GEMMELL & SOULSBY, 1968; RICKARD & WILLIAMS, 1982). However from our own experiments we cannot say at what time after infection metacystodes become susceptible to the immune response, but it is likely to be after emergence from the liver (i.e. 2–3 weeks post infection).

In summary, liposome entrapped low speed supernatants of TpO sonicates gave as good protection against the development of rabbit cysticercosis as TpO emulsified in FIA. Functional oncospherical antigen(s) in *T. pisiformis* may be more effectively presented to the host immune system on the liposomes surface. Vaccination with the TpMcES from the mature metacystode (in liposomes or FIA) did not prevent infection, but appeared to be effective in destroying the more mature metacystodes.

#### ACKNOWLEDGEMENTS

This work was supported by the Wellcome Trust and the EEC (STD).

#### REFERENCES

- BANGHAM, A. D., STANDISH, M. M. & WATKINS, J. C. (1965) Diffusion of univalent ions across the lamellae of swollen phospholipids. *Journal of Molecular Biology*, **13**, 238–252.
- CRAIG, P. S. (1984) Circulating antigens, antibodies and immune complexes in experimental *Taenia pisiformis* infections of rabbits. *Parasitology*, **89**, 121–131.
- CRAIG, P. S. & RICKARD, M. D. (1981) Anti-oncospherical antibodies in the serum of lambs experimentally infected with either *Taenia ovis* or *Taenia hydatigena*. *Zeitschrift für Parasitenkunde*, **64**, 169–177.
- CROFT, S. L. (1986) Liposomes in the treatment of parasitic diseases. *Pharmacy International*, **7**, 229–233.
- FLISSER, A., PEREZ-MONTFORT, R. & LARRALDE, C. (1979) The immunology of human and animal cysticercosis: a review. *Bulletin of the World Health Organization*, **57**, 839–856.
- GEMMELL, M. A. & SOULSBY, E. J. L. (1968) The development of acquired immunity to tapeworms and progress towards active immunization, with special reference to *Echinococcus* spp. *Bulletin of the World Health Organization*, **39**, 45–55.
- RAJASEKARIAH, G. R., RICKARD, M. D. & MITCHELL, G. F. (1980) Density gradient separation of *Taenia pisiformis* oncospheres. *Journal of Parasitology*, **66**, 355–356.
- RAJASEKARIAH, G. R., RICKARD, M. D. & O'DONNELL, I. J. (1985) *Taenia pisiformis*: protective immunization of rabbits with solubilized oncospherical antigen. *Experimental Parasitology*, **59**, 321–327.
- RICKARD, M. D. (1982) Immunization against infection with larval taeniid cestodes using oncospherical antigens. In: *Cysticercosis: Present State of Knowledge and Perspectives* (editors A. Flisser, K. Willms, J. P. Lacleste, C. Larralde, C. Ridaura & F. Beltran): Academic Press: London, pp. 633–646.
- RICKARD, M. D. & WILLIAMS, J. F. (1982) Hydatidosis/cysticercosis: immune mechanisms and immunization against infection. *Advances in Parasitology*, **21**, 229–296.
- SIDDIQUI, W. A., KAN, S. C., KRAMER, K., CASE, S. & PALMER, K. (1981) Use of a synthetic adjuvant in an effective vaccination of monkeys against malaria. *Nature*, **289**, 64–66.
- SZOKA, F. & PAPAHAJOPOULOS, D. (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse phase evaporation. *Proceedings of the National Academy of Science, USA*, **75**, 4194–4198.
- TOM, B. H. (1980) An overview: liposomes and immunobiology—macrophages, liposomes and tailored immunity. In: *Liposomes and Immunobiology* (editors B. H. Tom & H. R. Six): Elsevier North Holland: Amsterdam, pp. 3–22.
- VAN ROOIJEN, N. & VAN NIEUWMEGEN, R. (1980) Liposomes in immunology: evidence that their adjuvant effect results from surface exposition of the antigens. *Cellular Immunology*, **49**, 402–407.

Accepted 13th May 1987.